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VOL. 23, SEC. C. FEBRUARY, 1945 NUMBER 1

THE CAUSE OF INCOMPATIBILITY BETWEEN BARLEY AND RYE[1]

BY W. P. THOMPSON[2] AND DOROTHY JOHNSTON[3]

Abstract

When common barley is pollinated by common rye fertilization takes place normally but no viable seeds are produced. The immediate cause of the failure is abnormal development of the hybrid endosperm and particularly of the endospermic nuclei, which from an early stage are of immense size and are always few in number. No cells are ever formed in the endosperm. It collapses on the fifth or sixth day after pollination and disintegrates. The hybrid embryo, on the other hand, although it grows more slowly than the pure barley embryo from the third day, remains normal and healthy in appearance long after the endosperm has become very abnormal or has completely collapsed. The behaviour of the maternal tissues of crossed material is similar to that of selfed material, and rules out the theory of somatoplastic incompatibility. The behaviour of the antipodal cells of the embryo sac is not sufficiently different from normal to be of significance.

Introduction

The exact nature of the breakdown in the reproductive processes of incompatible wide crosses has been determined in surprisingly few instances. But the available evidence (7) indicates that in a large proportion of such crosses among flowering plants fertilization takes place normally and that the fault lies in the hybrid endosperm. This is true both in those plants like the Gramineae in which the endosperm normally persists and forms a large part of the seed and in those in which it is an evanescent structure.

It is to be expected, of course, that the visible breakdown, which constitutes the immediate cause of the incompatibility, may often be a secondary effect of a more basic factor, and that a chain of events may intervene between primary and immediate causes. One such basic cause has been identified by Cooper and Brink (3) as unsuccessful competition of the endosperm with maternal tissues for a limited food supply. They find that in a number of interspecific crosses the maternal tissues of the ovule quickly proliferate after fertilization instead of regressing as in normal development, while the endosperm develops slowly and then disintegrates. They suggest that in normal development the endosperm maintains "an ascendant physiological position relative to adjacent maternal tissue," whereas in crosses it loses its ascendancy

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because of its slow development, and is starved by the hyperplasia of the maternal tissue. In other words there is competition for a limited food supply between maternal tissue and endosperm; normally the endosperm develops rapidly and gains the upper hand, but in interspecific crosses it develops slowly, loses the dominant position and collapses through starvation.

More recently the same authors (2, 4) appear to abandon this theory of somatoplastic incompatibility with respect to certain plants and to substitute as a basic factor an abnormal behaviour of the antipodal cells of the embryo sac. It is believed that these cells "function in the secretion of substances necessary for early endosperm growth." Normally they are stimulated at fertilization to marked hypertrophy, become extremely vacuolated, and then rapidly decline. On the other hand, these authors find that when *Hordeum jubatum* L. is pollinated by *Secale cereale* L. enlargement is limited, vacuolation is slight, and the cells persist and even divide. This behaviour is taken to indicate that the antipodals do not function properly in the crossed material and that therefore the hybrid endosperm fails to receive all the materials necessary for its growth. Its development is therefore arrested.

An interesting example of an intergeneric cross that fails because of a breakdown in the reproductive processes after successful fertilization is that between common barley and common rye. The fact that when rye pollen is applied to the stigma of barley a swelling of the ovary occurs was discovered by Quincke (6) who requested us to determine what had actually happened. One of us reported briefly on the development and abortion of the hybrid tissues at the Seventh International Genetics Congress in 1939 (8). Conditions connected with the war have prevented earlier publication of the full account. In the meantime Cooper and Brink (4) have described events in a cross between the wild barley, *Hordeum jubatum* L., and rye. (*H. jubatum* L. is tetraploid in contrast to common barley, *H. vulgare* L., which is diploid.) They have also (2) given some information on the antipodal cells of the embryo sac in *H. vulgare* pollinated by rye.

Materials and Methods

A number of different varieties of common barley were used as female parents in the cross pollinations, but no difference in the degree of success of the experiments with the different varieties could be discovered. In all the later work Regal barley was used. The variety of rye (*Secale*) that furnished the pollen was Prolific.

Ovaries were fixed at 1, 2, 3,. . . . . 14 days after cross pollination but it was found that the important events had all taken place by the eighth day and the description is largely confined to that period. All pollinations were made early in the morning and all fixations at the same hour on succeeding days. A corresponding series of barley ovaries artificially pollinated by barley was fixed for comparison. For simplicity the latter material will hereafter be called 'selfed,' in contrast to the 'crossed' material, although in

reality the barley pollen did not always come from the same plant as the ovary.

Reciprocal cross pollinations were also performed and the material studied in the same way. But no evidence of the development of either embryo or endosperm could be discovered when barley was the pollen parent.

Results

The Unfertilized Ovule and Ovary

The ovary of barley closely invests the single anatropous ovule (Figs. 1 and 2) and becomes incorporated in the coat of the mature grain. It must therefore be considered in relation to the theory of competition between maternal and hybrid tissues for the available food supply. It is somewhat flattened in a dorsoventral direction as shown in the cross section represented in Fig. 2. The placenta is on the ventral wall, which is somewhat thicker than the dorsal. Above the level of the ovule the ovary enlarges considerably to the base of the sessile stigma. The main vascular bundle (*v*, Figs. 1 and 2) runs through the ventral wall just outside the placenta. At the base of the ovary this bundle gives off two small strands, which diverge to either margin where they also run vertically.

The single ovule is without a stalk and is attached to the placenta by its side over a comparatively large area. It carries two integuments, each two layers of cells in thickness (*ii* and *oi*, Figs. 1, 2, and 3). The outer integument falls some distance short of reaching the micropyle. At the base of the ovule the nucellus is a considerable tissue but thins gradually to a single layer at the apex. This layer is the epidermis, which is everywhere well differentiated.

The only special feature of the embryo sac is the large number (about 15) of conspicuous antipodal cells. These lie near the base of the sac on the ventral side against the placenta. They are filled with dense contents. The large number appears to be a common feature of the Gramineae.

Development of the Embryo

With the technique that was used in this work 90% of the self-pollinated barley flowers became fertilized and 80% of those pollinated by rye.

One day after pollination the egg is still undivided in the great majority of selfed barley flowers, although the pollen tube has entered the embryo sac. At two days nearly all embryos have only two cells (Fig. 4, A, bottom) but a few have three and very few, four. At three days (Fig. 9, A) there are usually about six cells but the number varies from four to 12; the average length of the embryo at this time is 0.06 mm. (Table I). Thereafter (Figs. 10 to 13) it grows rapidly and becomes pendant-shaped. At seven days, when it averages 0.4 mm. in length, the stem tip is evident and differentiation of tissues has begun.

At first the hybrid embryo keeps pace with the pure one or is slightly ahead of it. A somewhat higher proportion of the eggs has divided at one day and at two days more have passed on to the three- or four-celled stage, although

TABLE I

COMPARISON OF 'SELFED' AND 'CROSSED' EMBRYOS

Days after pollination	Number of cells				Mean length in mm.	
	Selfed		Crossed			
	Range	Usual	Range	Usual	Selfed	Crossed
1	Egg-2	Egg	Egg-3	Egg	—	—
2	2-4	2	2-5	2	—	—
3	4-12	6	2-6	4	0.060	0.060
4	10-30	16	4-10	6	0.072	0.063
5	16-60	24	8-12	10	0.085	0.068
6	Many	—	10-20	14	0.117	0.078
7	Differentiation	—	16-30	20	0.415	0.088
8	—	—	16-40	25	0.580	0.108

the majority have only two cells (Fig. 4, B). But by three days the hybrid begins to lag (Fig. 9, B); it has two to seven cells whereas pure barley embryos have five to 12. Thereafter divisions proceed slowly (Figs. 10 to 13) until at seven days there are only 16 to 30 cells (mean = 20), and an average length of 0.09 mm. At this time the pure barley embryo has a very large number of cells and averages more than four times as long. At this time also the selfed embryo has begun the differentiation of tissues, whereas the hybrid in no case becomes differentiated at this or any later time. From six days on most of the embryos are somewhat flattened by the pressure of the maternal tissue resulting from the collapse of the embryo sac. This may cause an increase in length without real growth.

Apart from their slower growth and flattening, however, the hybrid embryos appear perfectly normal up to seven days. But at eight days a proportion of them begin to appear unhealthy; some of their cells have vacuolated cytoplasm and dense, structureless nuclei. Thereafter degeneration proceeds and by 12 days all of them have lost their protoplasm and collapsed, or appear as faint masses in which the structure is difficult to observe. The largest number of cells observed was 40 and the greatest length 0.14 mm.

It should be noted that all embryos remain perfectly healthy until long after the associated endosperm has become highly abnormal, many of them until after the whole embryo sac has completely collapsed.

Development of the Endosperm

In nearly half of the selfed flowers the primary endosperm nucleus has divided at least once by the first day after pollination; in some it has divided twice and in a few, three times. Thus the first endosperm division precedes that of the egg, and subsequent divisions proceed more rapidly than in the embryo. For a short time the daughter nuclei remain in the region between egg and antipodals but soon distribute themselves through the peripheral layer of cytoplasm.

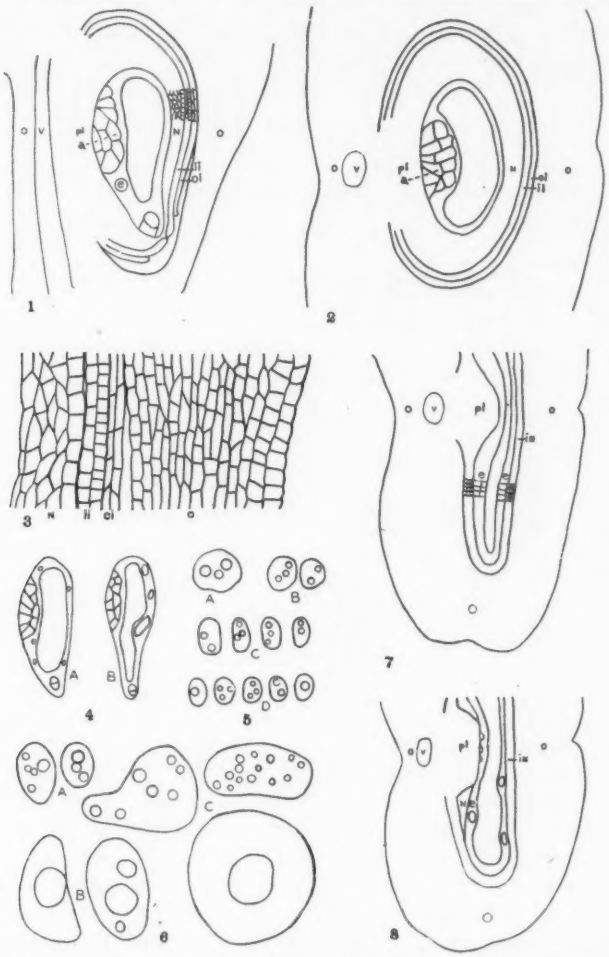


FIG. 1. *Median longitudinal section of unfertilized ovule and part of ovary of barley,* × *60; o, ovary wall; v, vascular bundle; pl, placenta; a, antipodal cells; n, nucellus; ii, inner integument; oi, outer integument; e, endosperm nucleus. (The labels carry the same meaning in the other figures.)*

FIG. 2. *Cross section of barley ovule and part of ovary at one day after pollination.* × *60.*

FIG. 3. *Strip of tissue from longitudinal section of unfertilized barley, extending through dorsal wall from surface of ovary (right) to embryo sac.* × *230.*

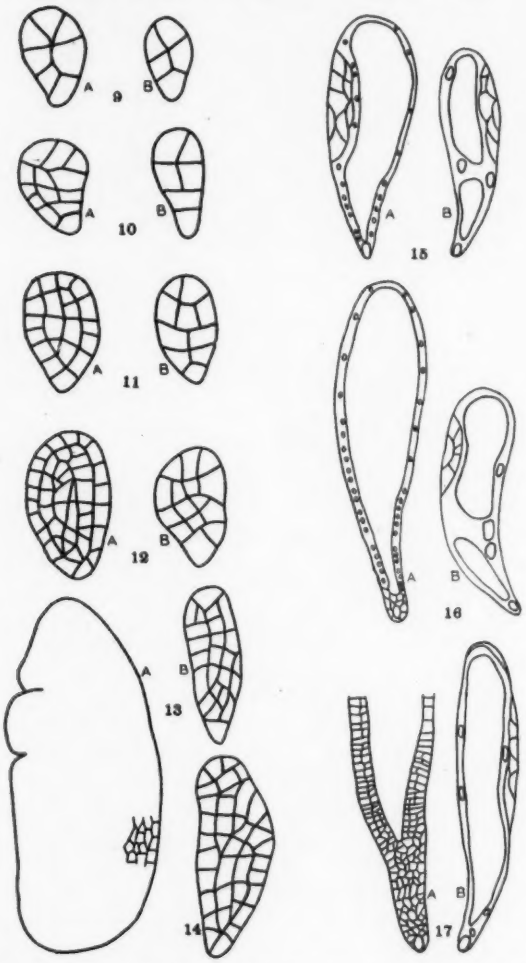
FIG. 4. *Embryo sacs at two days after pollination. A, from selfed flower; B, from flower pollinated by rye.* × *30.*

FIG. 5. *Nuclei from endosperm of selfed barley. A, fertilized primary nucleus; B, the first two nuclei; C, the first four; D, typical nuclei from multinucleate sac of the fourth day.* × *230.*

FIG. 6. *Nuclei from endosperm of barley pollinated by rye. A, the first two; B, two of the first four; C, representative nuclei from later stages.* × *230.*

FIG. 7. *A little more than half of a cross section of a four-day selfed ovary.* × *30. The endosperm is more advanced than in most four-day ovules.*

FIG. 8. *A similar section of a crossed ovary.* × *30.*



FIGS. 9 to 13. *Embryos at three, four, five, six, and seven days. A, pure barley; B, hybrid.* × *230, except 13, A, which is* × *130.*

FIG. 14. *Hybrid embryo at eight days.* × *230.*

FIGS. 15 to 17. *Embryo sacs at three, four, and five days. A, selfed; B, crossed.* × *30. In 17, A, only the lower half is shown.*

By two days (Fig. 4, A) there are from 16 to 128 free endosperm nuclei and by three days (Fig. 5, A) 200 to 520. On the fourth day (Fig. 16, A) the pocket around the embryo has become cellular and in some cases cells have also formed in the peripheral layer of cytoplasm. By five days (Fig. 17, A) the endosperm consists of a completely cellular layer surrounding a central cavity; usually this layer is two or three cells in thickness on the placental

side and one cell thick elsewhere. The cavity has become completely filled with cells on the sixth day, and on the seventh starch grains appear in the cells. The increase in length of the whole sac is shown in Table II.

TABLE II

COMPARISON OF SELFED AND CROSSED ENDOSPERMS

Days from pollination	Average length of embryo sac in mm.		Number of nuclei		Other points	
	Selfed	Crossed	Selfed	Crossed	Selfed	Crossed
1	0.52	0.62	1-8	1-8	—	—
2	0.63	0.67	16-128	1-16	—	—
3	0.95	0.92	200-500	2-30	—	—
4	1.35	1.08	Many	Up to 30	Cells	—
5	2.19	1.14	—	Up to 30	All cellular	Some collapsed
6	2.80	1.51	—	Up to 30	Cavity filled	Majority collapsed
7	4.74	1.58	—	—	Starch	—
8	5.13	1.59	—	—	—	All collapsed

In crossed material the first divisions of the primary endosperm nucleus take place as soon as or a little sooner than in selfed material but after one day the divisions proceed much more slowly. The largest number of hybrid endosperm nuclei seen at two days is 16, whereas in pure barley there may be as many as 128 at that time. The number ultimately produced varies from ovule to ovule but even at the latest stages before the collapse of the embryo sac it rarely exceeds 20.

The enlargement of the whole embryo sac keeps pace with that of selfed material up to three days but thereafter it lags behind and in fact grows comparatively little. From the third to the eighth day it increases in length only from 0.92 to 1.58 mm., whereas the selfed sac increases during the same period from 0.95 to 5.13 mm. (Table II). It should be noted, however, that up to the third day there is no reason for it to lose its ascendancy over the maternal tissue, and by that time great internal irregularities have already appeared.

The most striking feature in the development of the hybrid endosperm is the very abnormal behaviour of the nuclei. Even the two that result from the first division of the primary endosperm nucleus are larger than normal, and in some cases are very much larger. At two days some of them are larger than the whole embryo. They tend to increase progressively in size and at the end nearly all are huge. Compare Fig. 5 (selfed) with Fig. 6 (crossed). In some cases the diameter of the hybrid nuclei is eight times that of selfed ones.

In addition they assume a variety of shapes. Usually somewhat elongated in accommodation to the thinness of the cytoplasmic layer, they may also be spherical, dumb-bell–shaped or slipper-shaped, rectangular, or with protrusions of different forms.

The nucleoli vary greatly in number and size. At first most endosperm nuclei have three nucleoli, as do those of selfed material, but in later stages the number ranges from one to 20. When the number of nucleoli is small their size is very great, sometimes greater than that of a whole normal nucleus. The large ones contain one or more vacuoles.

Only three division figures have been observed in the hybrid endosperm. All of them show a very large number of chromosomes. The number cannot be determined accurately but appears to be at least 100. Since in both barley and rye the haploid number of chromosomes is seven, the expected number in the hybrid endosperm is 21. The division figures are very irregular with many chromosomes lagging at anaphase or forming bridges or clusters between the poles.

From the size, number, and appearance of the nuclei as well as from the division figures, it may be concluded that the chromosomes or many of them have multiplied several times without regular mitotic division.

As would be expected from these nuclear irregularities, the development of the whole endosperm is very abnormal. After three days there is very little growth. In some sacs the cytoplasm is fairly abundant in the micropylar pocket or near the antipodals but is elsewhere a very thin strand. *Cells are never formed.* Some of the nuclei begin to disintegrate at four days, but there is variation in this respect. At five days some of the embryo sacs have begun to collapse. By six days the majority have completely collapsed, with the two sides in contact except where held apart by the embryo. By eight days the collapse is complete in all sacs.

Behaviour of the Antipodal Cells

The first draft of this paper contained no account of the antipodal cells of the embryo sac other than the reference in the section on the unfertilized ovule, because nothing sufficiently notable had been observed. Since then Brink and Cooper (2) have attributed to them a very important role in the failure of wide crosses in the Gramineae. A careful re-examination of them has therefore been made.

Like other Gramineae, *Hordeum* has a large number (about 15) of antipodals, which constitute a conspicuous feature of the mature, unfertilized embryo sac (Figs. 1 and 2). They lie on the ventral side against the placenta near the base but since the growth of the sac is greatest at the base they subsequently assume a more median position. They are large cells with dense contents, irregularly fitted together.

Following self-fertilization they increase gradually in size until on the fourth day the whole mass is about twice as long as before (Table III). There is little or no increase in thickness of the whole mass so that the individual cells appear longer but no wider. There is no increase in the number of cells. The cytoplasm becomes more and more vacuolated until by the fourth day vacuoles occupy most of the volume. The cells become greatly flattened and crushed. By the fifth day in most cases the antipodals have become a

thin collapsed strip in which the boundaries of the individual cells are difficult or impossible to distinguish. These changes are to be expected as a result of stretching and pressure and the use of the materials of the antipodals with the rapid growth of the endosperm. They are similar to the changes in the nucellus.

TABLE III

LENGTH OF THE MASS OF ANTIPODAL CELLS IN MM.

Day	Selfed	Crossed
Unfertilized	0.24	
1	0.27	0.29
2	0.30	0.32
3	0.39	0.33
4	0.48	0.37
5	Collapsed	0.35

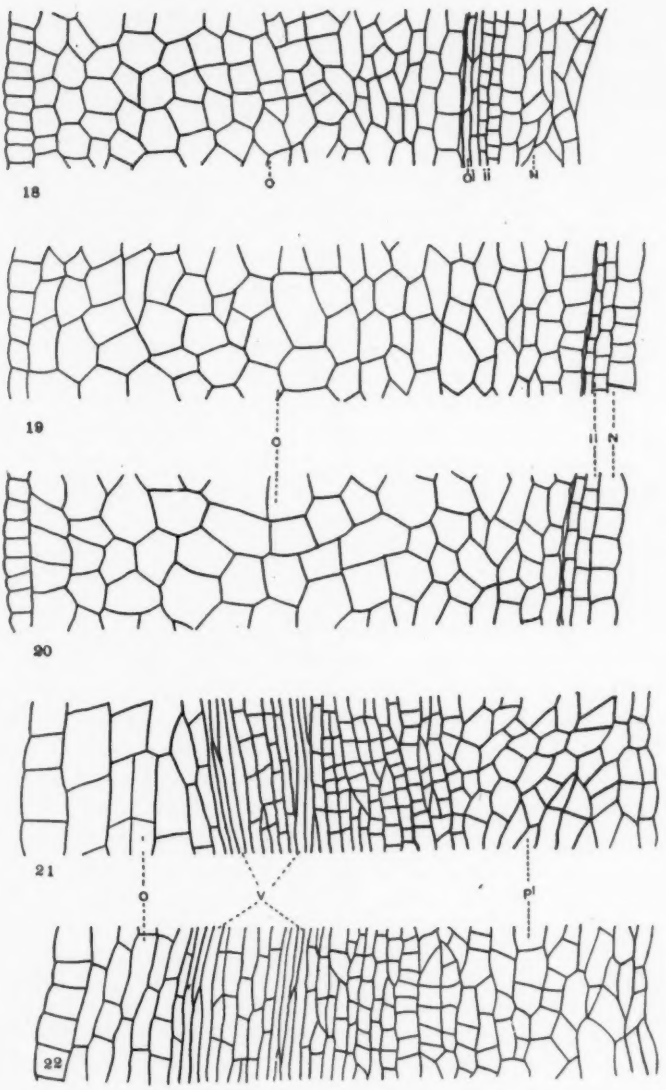
In the crossed material the behaviour of the antipodals is similar but not so pronounced. At first they increase at the same rate as those of selfed material but by the third day (Table III) the whole mass is somewhat shorter and there is little increase in its length thereafter. On the other hand they do not become so flattened. They also become vacuolated though not to such an extreme degree as do those of selfed material. They never increase in numbers by mitosis as do those of *H. jubatum* pollinated by rye (2). They persist longer than those in *H. vulgare* selfed, though some have collapsed by the fifth day.

In general the changes in the antipodals of crossed material differ from those of selfed in a way to be expected in view of the slower growth of the embryo sac and failure of the endosperm.

Maternal Tissues

The wall of the mature unfertilized ovary (Figs. 1 and 2) is thinnest on the dorsal side in the median plane at the base and increases both vertically and laterally. From about 10 cells in thickness at the bottom it becomes more than 20 cells thick at the level of the base of the ovule (which is uppermost). There is a well differentiated outer epidermis, then several layers of relatively large, irregularly arranged cells that grade into small ones at the inside. The two innermost layers have denser contents. Including the placenta, the ventral wall is not only considerably thicker but of a more uniform thickness. Through it runs the main vascular bundle. Between this bundle and the placenta is a zone of small meristematic cells that, in later stages, adds to the thickness of this region.

Fig. 3 represents a strip of tissue extending from the outside of the dorsal wall of the ovary through all maternal tissues (ovary, integuments, and nucellus) to the embryo sac (left). It is from a longitudinal section of an unfertilized ovary. Fig. 18 is from a cross section of the ventral wall of a



FIGS. 18 to 20. *Cross sections of maternal tissues of ventral side of ovary. In each figure the strip extends from outer surface of the ovary (left) to inner surface of nucellus (embryo sac) and is located between the placenta and the edge of the embryo sac.* × 230. *Fig. 18, two days selfed. Fig. 19, four days selfed. Fig. 20, four days crossed.*

FIGS. 21 to 22. *Longitudinal sections of ventral wall, including the placenta. The strips extend from the outer surface of the ovary to inner surface of placenta.* × 230. *Fig. 21, selfed. Fig. 22, crossed.*

two-day ovary. The strip of tissues represented is from the region at one side of the placenta, and extends from the outside of the ovary to the embryo sac. Similar strips from the same region of four-day ovaries are represented in Figs. 19 and 20, the former selfed and the latter crossed. The absence of any essential difference between them is obvious. Fig. 21 is from a longitudinal section of a selfed four-day ovary and passes through the middle of the placenta. The vascular bundle and the meristematic tissue between it and the placenta may be observed. Fig. 22 is a corresponding section of a crossed ovary of the same age. In this region also it is clear that there is no important difference between selfed and crossed material.

TABLE IV

COMPARISON OF SELFED AND CROSSED OVARIES WITH RESPECT TO THE THICKNESS OF THE WALL (IN MM.)

Day	Ventral, including placenta		Dorsal		Lateral	
	Selfed	Crossed	Selfed	Crossed	Selfed	Crossed
1	0.24	0.29	0.16	0.18	0.37	0.37
2	0.32	0.37	0.24	0.27	0.39	0.37
3	0.42	0.42	0.29	0.27	0.37	0.35
4	0.50	0.46	0.30	0.29	0.35	0.29
5	0.54	0.37	0.27	0.22	0.29	0.16

Table IV gives the thickness in mm. of the ovary wall at three points on successive days. The measurements are means of several ovaries at each age. They were made on cross sections as nearly as possible at the level of the middle of the embryo sac. One point chosen is the ventral wall at the greatest thickness of the placenta; the second is the dorsal wall directly opposite the first, and the third is midway between the other two. The differences between the selfed and crossed ovaries of the first two days are of no significance. The placental wall of selfed ovaries increases regularly throughout the period, as is to be expected in view of the necessity for feeding the large and growing endosperm and embryo. The same wall of crossed ovaries keeps pace up to the third day, but then lags and indeed shrinks when the endosperm collapses on the fifth day. The dorsal wall, after a much smaller amount of growth shrinks in both. The shrinkage of the lateral wall is pronounced, particularly in crossed material. Clearly there is no overgrowth of the wall of crossed material to support a theory of competition with the endosperm.

Nor can any real difference be seen in the make-up of the ovary wall. An increase in the density of the contents of the two innermost layers takes place in both selfed and crossed material. The vascular bundles remain similar in the two.

The outer integument is from the first in close contact with the ovary wall except at the tip of the ovule. It is composed (Fig. 3) of two layers of flat,

thin-walled cells with lightly staining cytoplasm. Up to two days no change is evident in this integument in either selfed or crossed ovules (Fig. 18). In both it collapses on the third day, apparently crushed by the growth of the embryo sac, and by the fourth day has disappeared in both or is represented by a thin line (Figs. 19 and 20).

The inner integument is composed of two layers of shorter, more cubical cells than the outer. It persists and undergoes no essential change in either crossed or selfed material during the whole period covered by this study (Figs. 19 and 20).

The nucellus of the unfertilized ovule (Figs. 1 and 3) is a considerable tissue at the base but becomes thinner towards the apex where it consists of a single layer that is continuous with the epidermis. With the growth of the embryo sac it gradually disappears. In selfed material of the second day there are two or three rows of loose, irregular cells plus the epidermis on the dorsal side and somewhat more on the ventral side at the right and left of the placenta (Fig. 18). By the third day it is reduced to one or two rows of collapsed cells and the epidermis on the dorsal side, and little more on the ventral. By the fourth day (Fig. 19) only the epidermis remains except against the placenta where some crushed remnants can usually be seen. The epidermis then resembles a third layer of the inner integument and might be so regarded if its earlier history were not known.

In crossed ovules the changes in the nucellus are similar to those in selfed ones. The only discernible difference is that, in accordance with the slower growth of the embryo sac, they frequently do not take place so rapidly and remnants persist longer on the ventral side next to the placenta (Fig. 8). But by the fifth day all the nucellus except the epidermis has disappeared (Fig. 20).

Discussion

The impression appears to be general that the breakdown in the reproductive processes in unsuccessful wide crosses usually takes place before fertilization and is due to the failure of the pollen to germinate or its inability to fertilize the egg. The number of crosses are, however, few in which the exact stage and nature of the breakdown has actually been determined. And the fact that fertilization is accomplished normally and considerable hybrid development occurs in the cross between two such distinct genera as barley and rye, leads to the suspicion that the difficulty arises after fertilization much more commonly than has been supposed.

In this connection it is important to note that the genera *Triticum*, *Aegilops*, *Agropyron*, *Haynaldia*, *Secale*, and *Hordeum* have been intercrossed successfully in a large number of combinations and that in other combinations fertilization has been demonstrated. This unusual body of results may be due to an unusual taxonomic situation, but is more probably due to the fact that since three of those genera are of great economic importance, much more genetic work has been done with them than with other groups of genera. It

is to be noted also that the economically very important genus *Zea* has been crossed with two others, *Euchlaena* and *Tripsacum*, and that fertilization has been shown to take place when much-studied *Nicotiana* is pollinated by *Petunia*, *Salpiglossis*, *Nicandra*, or *Solanum*. Therefore it seems probable that intensive work would reveal many hitherto unsuspected cases in which interspecific or intergeneric crossing is possible and a great many in which failure to cross is due to postfertilization breakdown.

In the case reported in this paper it is clear that the immediate cause of the failure is the irregularities in the hybrid endosperm and particularly in the endospermic nuclei. The embryo remains healthy long after gross irregularities have appeared in the endosperm, and indeed, with the exception of its reduced size, appears normal until after the endosperm has aborted and the whole embryo sac has collapsed. The endosperm has also been shown to be at fault in several other interspecific crosses in the Gramineae (1, 9) but the irregularities were much less extreme than those reported in this paper. Perhaps the endosperm plays an unusually important role in the Gramineae, since in that family it normally persists and forms a large part of the ripe seed. But a similar situation has been found by Cooper and Brink (3), Ledingham (5), and others in different Dicotyledones in which the endosperm is an evanescent structure.

Cooper and Brink (3) agree that the breakdown of the endosperm is usually the decisive factor throughout flowering plants, but maintain that it is a secondary effect of competition with adjacent maternal tissue. (See Introduction for a statement of their theory of somatoplastic incompatibility.) From the observations reported in the present paper, however, it is clear that this theory is untenable in respect to the cross between barley and rye. The maternal tissues of crossed ovaries do not proliferate; in fact they behave in the same way as those of selfed ones until after the endosperm has collapsed. In his considerable experience with other wide crosses in the Gramineae including *Triticum*, *Aegilops*, *Agropyron*, and *Secale*, the senior author had never noted any effect of the maternal tissue, and a re-examination of preparations made in earlier investigations on those genera has confirmed the impression that in several wide crosses the behaviour of the maternal tissues is the same in crossed as in selfed material. Moreover the authors of the theory of somatoplastic incompatibility appear in their latest papers (2, 4) to have abandoned the theory for the cross between *Hordeum jubatum* and *Secale cereale* and by inference for the other Gramineae. This naturally raises two questions: Would a study of interspecific crosses in other families require an abandonment of the theory in respect to many of them also? May not the proliferation of the maternal tissue in those genera in which it has been observed be the result rather than the cause of the endospermic irregularities? The slow growth and abnormal condition of the endosperm may cause an excess of unused food materials, which would stimulate the growth of the adjacent sporophytic tissue.

In place of their theory of somatoplastic incompatibility Brink and Cooper (2) have recently substituted, with respect to *H. jubatum* pollinated by *S. cereale*, the suggestion that abnormal behaviour of the antipodal cells is responsible for the breakdown of the endosperm. The abnormalities consist of (i) much less enlargement in comparison with selfed material, (ii) only slight vacuolation, and (iii) their persistence and even mitotic division. This behaviour is taken to indicate that the antipodals are not carrying on their function of supplying food to the endosperm. (See Introduction for a fuller statement.)

The observations reported in the present paper give little or no support to this suggestion. The behaviour of the antipodal cells in *H. vulgare* pollinated by rye is not very different from that in barley selfed. For two days they increase in size at the same rate (which is not great) and never lag far behind. They do not become so elongated but also remain wide; presumably this difference in shape is due to the reduced enlargement of the embryo sac and the lack of pressure from a developing endosperm. They too undergo vacuolation though not to the same degree. They persist somewhat longer but collapse only a day or two after those of selfed ovules. They never divide as in *H. jubatum*. In general the behaviour of the antipodals in crossed material differs from that in selfed only slightly and in ways to be expected in view of the slower growth of the embryo sac and the failure of the endosperm. In other words the difference appears to us to be the effect rather than the cause of the events in the endosperm. This conclusion is strengthened by the consideration that the antipodals are not of hybrid constitution but have only maternal chromosomes, and therefore should not be expected to show hybrid incompatibility as should the endosperm.

If the breakdown of the endosperm cannot be attributed to simple competition with the maternal tissue for food or to abnormal behaviour of the antipodal cells, it must be regarded as due to the constitution of the endosperm itself or to an unfavourable reaction of some kind with the mother plant, resulting from that constitution. It has been argued that if the genoms of two distinct species or genera can work together satisfactorily in a hybrid embryo, they should prove equally compatible in the endosperm. But the chromosomal and genic conditions are not the same in the endosperm as in the embryo. Owing to the triple fusion in the origin of the primary endosperm nucleus the important matter of genic balance may be involved. When there are two barley chromosomes or genes of a particular kind to one of rye, there may be present an unbalanced condition that does not occur when there is one of each as in the embryo.

It has been suggested that some kind of immunity reaction may take place between the mother plant and the hybrid tissues which are of a very different genetic constitution. Or the mother plant may not produce the right kind or amount of food material for a hybrid with a different constitution. In either of these cases it is to be expected that the embryo would be more injuriously affected than the endosperm since it has a higher proportion of

non-maternal genes and chromosomes. But this latter consideration might be nullified if the embryo secures all its materials by way of the endosperm and not in unmodified form from the mother.

An important fact is that in barley pollinated by rye the initial abnormality of the endosperm and the most conspicuous one throughout is the condition of the nuclei. Even at the two-nucleate stage both may be of immense size. Perhaps the ultimate breakdown is due to inability to carry through mitosis properly with the resulting greatly increased number of chromosomes. Probably more important than the number is the irregular distribution of the chromosomes. But these may both be effects of a more basic condition of unbalance. The nuclei are abnormal in other cases in the Gramineae in which incompatibility or sterility must be attributed to the endosperm, but the great size and large number of chromosomes have not been reported in those cases.

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STUDIES ON THE *FUSARIUM* OF MUSKMELON WILT

I. PATHOGENIC AND CULTURAL STUDIES WITH PARTICULAR REFERENCE TO THE CAUSE AND NATURE OF VARIATION IN THE CAUSAL ORGANISM[1]

By John J. Miller[2]

Abstract

During the past decade a serious disease of muskmelon has appeared in Ontario. From diseased plants a *Fusarium* has been isolated, which proved to be highly pathogenic to muskmelons in infection experiments. Results indicate that the wilt organism occurs in nature as a form (or forms) that, when cultured on most artificial media, produces abundant aerial mycelium and this has been termed the 'wild type.' Mutation toward loss of aerial mycelium and darkening of the submerged mycelium is frequently observed in culture and, since many of the mutants are more aggressive than the parent, they tend to displace it. Evidence is presented that the wild type is genetically very stable in its natural environment. Since, moreover, certain of the commoner mutants caused less disease incidence under field conditions than the wild type, it is considered that variation of the sort observed in culture, if it does occur in the field, is unlikely to be of importance in the field pathology of the disease. The stability of the wild type in sterilized soil and soil agar indicates that these substrates may be of value for maintaining Fusaria in the pure state. Mutations were induced by irradiating spores with ultra-violet light. In general, these resembled those that appeared spontaneously and both groups were less pathogenic than the wild type. A phenomenon termed 'cultural interaction' was observed between certain of the mutant cultures and the wild type. Since this appeared to be of a specific nature, it may be of some taxonomic value in diagnosing species of *Fusarium*. The suggestion is offered that the concept of the 'wild type' may be of general application throughout the genus *Fusarium*.

Introduction

During the past decade a serious disease of muskmelons has appeared in Ontario. It was first reported about 1937, although the disease was probably present earlier than this. The field symptoms involve a wilting of the vines or, when less severe, a general unthriftiness and failure to mature marketable fruit.

From diseased vines has been isolated a *Fusarium* that proved to be highly pathogenic to muskmelons. In inoculated soil under experimental conditions, the typical symptom noted was a seedling wilt, which usually began after the cotyledons reached their full size. When seeds were planted in heavily infested soil, however, a seed rot or pre-emergence blight resulted and most of the seedlings that escaped this were found to damp off shortly after emerging.

Leach and Currence (9) have reported a Fusarium wilt of muskmelons in Minnesota, the causal organism of which they describe as *Fusarium bulbi-*

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Contribution from the Department of Botany, University of Toronto, Toronto, Ont. An abridgement of a thesis presented in May, 1944, to the University of Toronto in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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genum Cke. & Mass. var. *niveum* Wr. f. 2. Munger (12) states that the disease has become increasingly destructive during the past 10 years in New York State, particularly in the counties bordering on Lake Ontario. Gregory (7) reported Fusarium wilt of muskmelons in Indiana. Muncie (11) has reported the disease in Michigan and Middleton (10) in California. A wilt of muskmelons in Astrakhan has been reported by Rodigin (15) who states the causal organism to be *Fusarium reticulatum* Mont.

Materials and Methods

The cultural medium most frequently employed was potato dextrose agar prepared according to the formula of Riker and Riker (14) except that the content of agar was lowered from 1.7 to 1.5%. Soil infestation was accomplished by the addition of cornmeal–sand medium, the ingredients of which were mixed in the following proportions:—1000 gm. sand: 200 gm. cornmeal: 250 cc. water. Measured quantities were placed in flasks and autoclaved. For a given experiment the flasks were inoculated with spores and mycelium of the isolates and strains involved. They were shaken every two or three days to ensure uniform growth throughout and after 8 to 12 days the inoculum was considered ready for incorporation into the soil. In the earlier experiments the medium was added in the proportion of 2% by volume but this was later reduced to 1%.

The soil employed for most of the pathogenicity comparisons was obtained from one of the sandy knolls that appear irregularly throughout the Vineland Clay Loam area and it will be referred to here as 'sandy knoll' soil. This is a light soil that drains well and that resembles in texture the soil from melon fields in the infested areas. Two sizes of flats were employed throughout these experiments, a small size, 8 × 11 in. containing three litres of soil and a larger size, 11 × 18 in. containing 10 litres. In each of the smaller flats 75 seeds were planted and 140 in the large size. Since experiments seldom lasted more than 20 days, the advantage of working with large numbers was thus obtained without crowding.

The host variety selected for these experiments was Bender's Surprise, a large-fruited, vigorous melon with coarse, heavy vines. It was found to be highly susceptible to *Fusarium*.

The technique employed for isolating single spores was as follows: a conidial suspension was first made by adding sterilized distilled water to a slant culture. This was shaken to dislodge the spores and the suspension was then poured over the surface of potato dextrose agar in a Petri dish. Excess liquid was drained off by inverting the dish. When isolations were to be made from Petri dish cultures, a small amount of fungus material was removed and shaken with sterile water in the same manner. It was frequently necessary to dilute the suspensions to secure the desired density of spores on the plates. These were allowed 10 to 20 hr. for germination and then single germinating spores were removed, employing the 'biscuit-cutter' technique as described

by Keitt (8) except that no 'spatula' was found necessary. Thus a small piece of agar was transferred with the spore to a plate of potato dextrose agar on which the spores were spaced as desired.

Description of the Pathogen

A pathogenic *Fusarium* was isolated from diseased melon plants and also, on several occasions, from seedlings growing in naturally infested soil. This organism grew well in culture on most media tested, producing abundant white aerial mycelium with a rather flocculent appearance. In alkaline and in slightly acidified potato dextrose agar the colour of the submerged mycelium was a light cream. On a more acid medium this became a light vinaceous red and at pH 2.5, where growth was very slow, the cultures were a deep rusty red colour.

Occasional dark blue sclerotia are produced and observations have indicated that these are formed more abundantly when cultures are grown in the light than in darkness.

When the organism is grown on potato dextrose agar, non-septate conidia (microspores) are borne on the aerial mycelium but not abundantly and very few septate conidia (macrospores) are produced. Examples are shown in Fig. 1. Microspores from a monosporous culture on potato dextrose agar

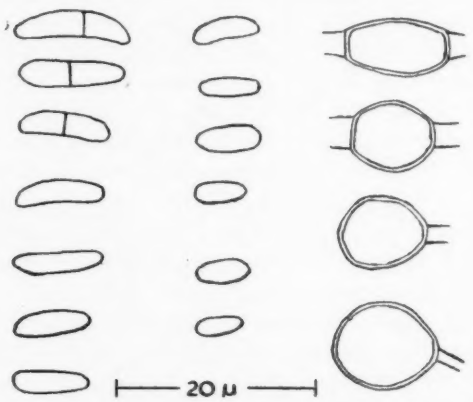

FIG. 1. *Left: Conidia produced on potato dextrose agar by a freshly isolated wild type culture. Right: Chlamydospores produced by the wild type on soil agar. Camera lucida drawings.* ×1500.

varied from $4.1 - 13.6 \times 1.4 - 2.7\mu$ with a mean of 6.4×1.8. Sporulation was similar on other agars, including malt, pea, beef-extract–peptone, synthetic (agar plus modified Pfeffer's nutrient solution with dextrose as the carbon source). On soil agar growth was sparse and very few conidia were produced, but chlamydospores were fairly numerous (Fig. 1). These were very infrequent on the other kinds of agar. On potato agar without added

carbohydrate, however, abundant sporulation was observed on the aerial mycelium. The conidia varied from non-septate to three-septate and have been found to occur in the following frequencies: non-septate, 76%; one-septate, 20%; two-septate, 3.5%; three-septate, less than 1%. The range in size of these spores in micra was as follows:

Non-septate, $4.4 - 10.9 \times 1.6 - 2.7$, mean 7.2×2.1
One-septate, $8.2 - 17.7 \times 1.8 - 2.7$, mean 12.1×2.5
Two-septate, $10.9 - 20.2 \times 2.4 - 3.0$, mean 15.4×2.6
Three-septate, $15.0 - 25.9 \times 2.7 - 3.0$, mean 18.9×2.8

Surface sporulation on diseased melons in naturally infested fields produced numerous three-septate macroconidia and a smaller number of spores with fewer septa. The mean size of these three-septate conidia was $17.9 \times 2.6\mu$.

Macrospores are frequently found in cultures on potato dextrose agar in limited areas where the mycelium is more compact and of a deeper colour when viewed from below. However, monosporous isolations from such areas have consistently yielded a high proportion of cultures that differ from the parent and are considered to be mutants. Many of the latter produce numerous macrospores on potato dextrose agar and one such culture yielded three-septate macrospores up to 42μ in length.

Initially all isolates obtained from plants diseased in naturally infested soil were found to be alike and to correspond with the preceding description. More than 70 such isolates have been obtained, involving plants from a number of melon fields and all were of this type. From one of these, however, three out of 12 single spores produced cultures that differed from the others in the character of the aerial mycelium. It is possible that this is another naturally occurring form of the melon pathogen and that the plant concerned was attacked by both, but on the other hand the new form may have arisen from the other by a mutation in the Petri dish culture before the single spores were isolated.

When the above description is compared with that given by Leach and Currence (9) for the organism causing muskmelon wilt in Minnesota, it is apparent that the spores were much larger in the Minnesota *Fusarium*. However, the spores they measured were taken from regions in the cultures where macrospores were abundant and, in view of the work described here, it is possible that this description may be based on one of the mutants of the original wild type. Although various mutants have been obtained in the course of this work, it is not possible to know which of these may correspond to the mutant of the Minnesota *Fusarium* and hence it seems unsafe to attempt a comparison. However, from other points of their description it is evident that the two organisms have certain features in common. Both possess white aerial mycelium, are bright red on very acid media, and produce blue sclerotia in old cultures. From these similarities it seems hardly reasonable to assume that the organisms are identical but, as Nelson *et al.* (13) state: "the host relationship appears worthy of recognition in classifying the pathogenic forms of Fusarium." They point out, for instance, that *Fusarium*

conglutinans Wr. cannot be distinguished from its variety *callistephi* Beach except by inoculation tests. Hence the Ontario muskmelon *Fusarium* is considered to be closely similar to that described by Leach and Currence as *Fusarium bulbigenum* Cke. & Mass. var. *niveum* Wr. f. 2.

Cytological Investigations

In view of the nature of the work that follows, it was found advisable to determine the nuclear situation in the mycelium and spores of the organism.

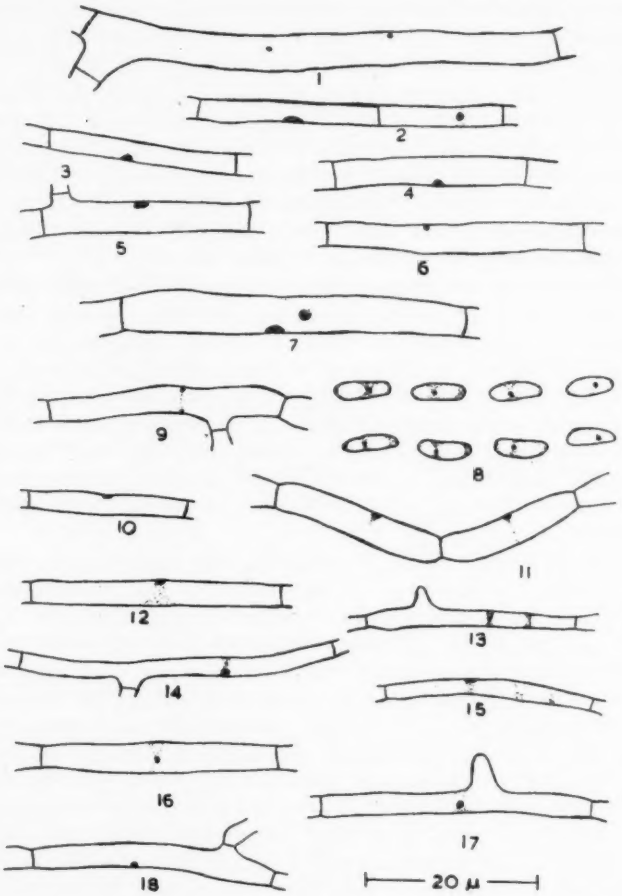

FIG. 2. *Camera lucida drawings of Feulgen stained mycelial cells and spores of the muskmelon wilt Fusarium.* ×1100. *1 to 7: Mycelial cells of a light appressed mutant. Nos. 1 and 7 are binucleate. 8: Microspores produced by the wild type. 9 to 18: Mycelial cells of the wild type. No. 9 is binucleate.*

For this purpose the Feulgen stain technique was employed. Material for staining was prepared by inoculating thin films of potato dextrose agar on slides with spore suspensions of two strains of the organism under sterile conditions. The slides remained for four days at 27° C. in Coplin jars that contained moist filter paper to prevent drying. They were then fixed in Carnoy 3 : 1 fixative for five minutes, following which they were Feulgen stained, employing the technique developed in the Department of Botany, University of Toronto, by Dr. L. C. Coleman and his associates (2, 3).

It is apparent from Figs. 2 to 6 that most of the mycelial cells and microspores were uninucleate. A few of the cells, however, were multinucleate, possessing two and sometimes three or four nuclei. It is interesting that more multinucleate cells were found in the mycelium of the light appressed mutant strain than in the parent wild type strain. No microspores were found with more than one nucleus. Macrospores were not formed by the wild type under these conditions but a number of one-septate macrospores were produced by the light appressed strain and in these each cell was observed to contain a single nucleus.

Mutation of the Organism in Culture

1. Cultural Degradation

When monosporous isolations are made from an isolate of the pathogen derived from a plant diseased in naturally infested soil, it is found as a general rule that all the cultures originating from single spores are alike and correspond to the description given in the preceding section. This cultural form has been termed the 'wild type.' If a slant of potato dextrose agar is inoculated from such a culture and transferred every week or two, it is commonly noted that after two months or less the culture in the slant has changed. If monosporous isolations are again performed, it is usually not found possible to reisolate the original but instead a different type of culture is obtained. Since it has been shown in the preceding section that the mycelial and spore cells are predominantly uninucleate, it seems reasonable to assume that a variant appearing in the culture originating from a single spore has arisen through mutation.

The process whereby the wild type is displaced by a mutant has been termed 'cultural degradation.' It is considered to be a degradation in the sense that the wild type is lost and the mutants tend to be less pathogenic than the parent. The mutants appear able to crowd out the parent type since they are more aggressive in culture. This is evidenced by the observation that mutants first appear as small dense patches of mycelium that increase in size. When first visible these patches are less than 1 mm. in diameter but they become wider attaining in some cases a diameter of 5 to 10 mm. (Fig. 7). They appear fairly early in the life of the cultures and have been observed after four days in cultures arising from a single spore. Monosporous isolations from such an area yield a high proportion of mutant cultures, all of which are usually of the same mutant type. It has not been found

possible to isolate mutants from adjacent normal regions. This indicates that the patches result from the invasion by a mutant of the surrounding region of the parent culture, a view that is supported by the fact that when certain mutant cultures make contact with cultures of the wild type, there is observed at the boundary between the two a phenomenon that has been termed 'cultural interaction.' This also appears to involve the invasion of the wild type culture by the mutant, since monosporous isolations made within the original boundary of the wild type yield a high proportion of cultures of the mutant. This will be discussed further under the heading of Cultural Interaction.

An additional factor favouring the loss of the wild type is that spores from certain mutant cultures germinate more rapidly than those of the wild type. For instance, it is found when making monosporous isolations from the 'patch mutants' that, if spores with long germ tubes are selected, the proportion of mutant cultures secured is greater than when the spores selected have relatively short germ tubes. Ninety spores with short germ tubes isolated from a total of seven such areas produced 30 mutant cultures, whereas 65 spores with long germ tubes isolated from the same areas produced 57 mutant cultures. Thus, when a mixed culture is transferred, the mutant would have an initial advantage as far as rapidity of germination of the spores is concerned.

2. Cultural and Pathogenicity Studies on Cultural Variants

(A) Description of Variants

Single spore analyses have yielded an apparently endless variety of new types, some of which are barely distinguishable from the wild type, but most show very striking differences. These variants may be classified roughly into a number of main groups on the basis of the depth of coloration of the submerged mycelium and the amount of aerial mycelium. Some of these are shown in Fig. 7. The light raised group, for instance, contains those cultures in which the submerged mycelium is light in colour and which have abundant aerial mycelium. The wild type is included in this group. Similarly, the dark appressed group includes those cultural types possessing dark submerged

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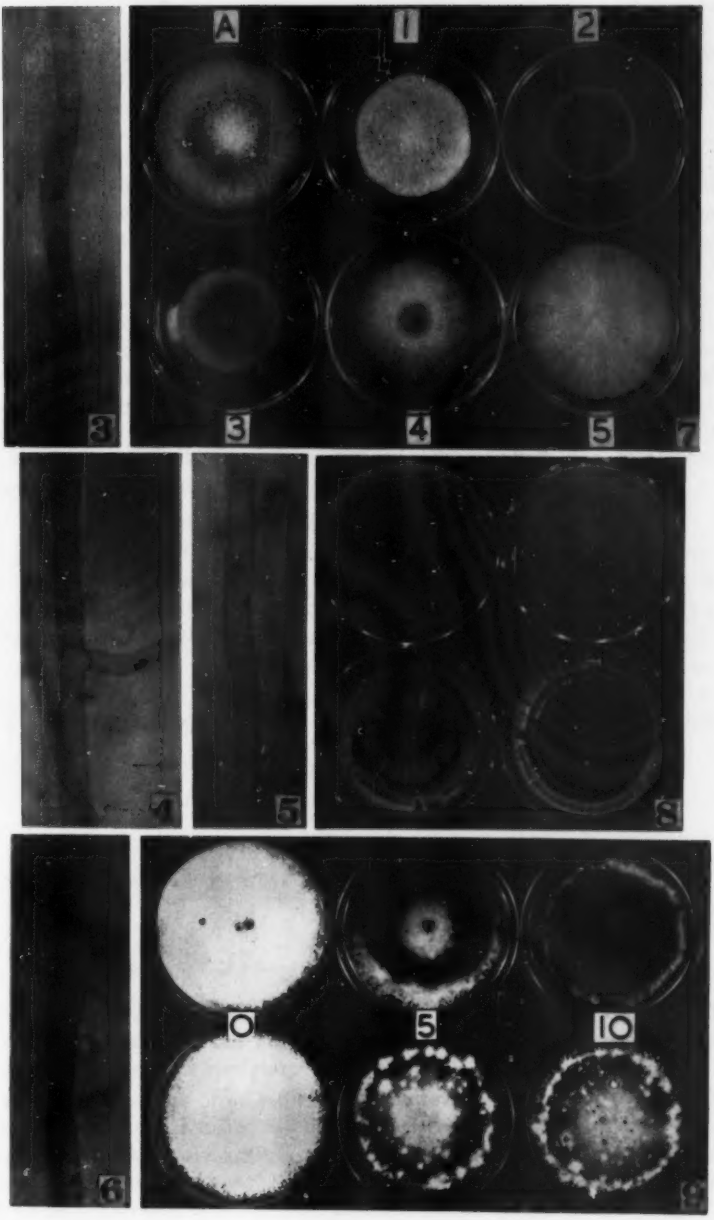
Explanation of Plate I

Figs. 3 to 6. *Microphotographs of Feulgen stained mycelial cells of a light appressed mutant of the muskmelon wilt* Fusarium. *×1200. The photographs were taken with a Leica Micca camera with a 10× Leitz Periplan ocular in conjunction with a 2 mm. Spencer apochromatic objective.* Fig. 7. *Eight-day-old cultures on potato dextrose agar of the wild type of the muskmelon wilt* Fusarium *and five mutant cultures. A = Wild type. Note the two 'patch mutants' near centre. 1 = Light raised. 2 = Dark semiraised. 3 = Dark appressed. 4 = Light appressed. 5 = Very light appressed.* Fig. 8. *Appressed mutants obtained by the single spore technique from patch mutants that appeared in irradiated cultures of the muskmelon wilt* Fusarium. Fig. 9. *The effect of irradiating growing cultures of the muskmelon wilt* Fusarium *with ultra-violet light for periods of five and 10 minutes. The cultures were 10 days old when irradiated and the photograph was taken 12 days later. The upper row of Petri dishes is inverted.*

PLATE I



mycelium and no aerial mycelium. In general, appressed cultures tend to sporulate more profusely than the raised type and the spores, on the average, are larger and have more septa.

(B) Pathogenicity Experiments

A total of 140 flats exclusive of checks have been inoculated and planted during the studies on the pathogenicity of the cultural variants. These were distributed throughout eight separate experiments. From these the general conclusion may be drawn that the mutants are less pathogenic than the wild type. There is, again, a considerable variation among the mutants in this respect. The dark semiraised group seemed only slightly less pathogenic than the wild type in flat experiments. The intermediate appressed is less pathogenic than the dark semiraised group and the dark and light appressed groups, again, are less pathogenic than the intermediate appressed. The least virulent of all groups was the very light appressed. The pathogenicity of all cultures in a given group was not consistent, which does not seem surprising since the classification is a purely artificial one.

(i) The Relation of Cultural Purification to Pathogenicity Studies

During the progress of this work it has been found that frequent monosporous purifications are necessary to maintain the wild type in a pure condition. If this precaution is neglected, the wild type is likely to be crowded out by mutants that, although more aggressive in culture, are less pathogenic.

In Table I are shown the values obtained for disease incidence with two cultures of the wild type in an experiment performed during the summer of 1942. Culture 1*A* was isolated from a seedling growing in naturally infested

TABLE I

Comparison of virulence of two freshly isolated wild type cultures

Culture	Wilt in 15 days, %	Wilt in 20 days, %
1*A*	62	79
2*A*	66	80

soil and 2*A* was obtained from a seedling that wilted in a flat of soil infested with 1*A*. It is evident from the table that both isolates seemed closely similar in pathogenicity at this stage.

In Table II the results of an experiment performed during the summer of 1943 are summarized. During the interim between these two experiments, Cultures 1*A* and 2*A* were maintained in culture on potato dextrose agar slants and were transferred every two months or so to avoid drying. Cultures 1*E* and 1*F* were recently isolated from plants diseased in the field.

From these tables it is evident that Culture 1*A* was not as virulent as it had been a year previously but Culture 2*A* compared favourably in this

respect with recent isolates of the wild type. An explanation for this was provided by monosporous analyses of the cultures. From 1*E* and 1*F* only the wild type could be isolated. In 1*A* the light appressed and dark appressed

TABLE II

COMPARISON OF THE VIRULENCE OF YEAR-OLD *Fusarium* ISOLATES, 1*A* AND 2*A*, WITH RECENT ISOLATES 1*E* AND 1*F*

Culture	Wilt in 15 days, %	Wilt in 20 days, %
1*A*	8	25
2*A*	94	100
1*E*	97	100
1*F*	96	100

types predominated and in 2*A* most of the isolates were dark semiraised. No isolates of the wild type were obtained from either tube. Since pathogenicity tests have shown that the appressed types tend to be much less virulent than the raised or semiraised, it is probable that the differences in virulence noted between 1*A* and 2*A* have arisen as a consequence of cultural degradation, which led to the predominance of different mutant types in two originally pure wild type cultures.

From this experiment it is evident that the ordinary laboratory procedure of serial transfers is not satisfactory for maintaining wild type cultures in a high state of purity and virulence. The question now arises, can this be achieved by successive monosporous purifications?

The results of an experiment performed to investigate this possibility are outlined in Table III. Inoculum of a number of wild type cultures was allowed to grow for eight days on cornmeal–sand medium, which was then incorporated in the proportion of 1% into sterilized 'sandy knoll' soil. In all cases except two, 1*E* and 1*F*, the flasks of medium were inoculated with freshly isolated single spore cultures. As usual, 140 seeds were planted per flat.

The first five cultures in the tables are sister single spore isolates from a wild type culture that had been maintained in the pure state for over a year by six successive monosporous isolations. The original 3*A* culture had been obtained in 1942 from 1*A* by passing the latter through the host twice. Cultures 2*Z* and *SC* lived over the winter of 1942-43 in soil. Culture 2*Z* was obtained in 1943 from a diseased plant growing in one of the greenhouses of the Horticultural Experiment Station at Vineland, Ont., the soil of which had been inoculated a year previously with an oat-hull culture of 1*A*. Culture *SC* was isolated in 1943 from a test-tube of 'sandy knoll' soil that had been sterilized and inoculated in 1942 with Culture 1*A*. The tube was allowed to become dry after the mycelium had grown throughout its contents and it was stored in a refrigerator at 4° to 5° C. during the winter. Subsequent to

TABLE III

COMPARISON OF VIRULENCE OF 12 WILD TYPE CULTURES OF THE MUSKMELON WILT *Fusarium*

Culture	Emergence, %	Wilt in 15 days, %	Wilt in 18 days, %
3*A*—6—*S*1	75	87	97
*S*2	75	90	96
*S*3	76	82	97
*S*4	78	85	96
*S*5	80	85	97
2*Z*—2	66	82	97
SC—2	77	86	98
1*D*—1	74	94	100
1*E*	77	92	98
1*E*—2	72	96	99
1*F*	69	82	96
1*F*—2	68	80	96
Check *a*	79	0	0
Check *b*	81	0	0
Check *c*	84	1	3
Check *d*	70	0	0

isolation, 2*Z* and *SC* were purified twice by the monosporous technique. Cultures 1*D*, 1*E*, and 1*F* were isolated from plants diseased in infested melon fields two months previous to this experiment. During this time 1*E* and 1*F* were purified twice and 1*D* once. The original cultures 1*E* and 1*F* are included without purification but care was taken to inoculate the flasks of medium from normal-looking parts of the slant cultures.

To check on the purity of the inoculum incorporated into the soil, the empty flasks were rinsed separately with sterile water and potato dextrose agar plates were inoculated with the suspensions obtained. In all cases a typical wild type culture resulted.

Of the four check flats, (*a*) was inoculated in the usual manner but with a non-pathogenic *Fusarium* and (*b*) with a non-pathogenic bacterium. Both had been isolated from diseased muskmelons. Flats (*c*) and (*d*) were untreated. There was considerable variation, from 66 to 80% in the emergence values throughout the inoculated flats but, since there was an almost equal variation in the checks, it is unlikely that the lower values were caused by pre-emergence blight. The wilt percentages were calculated on the basis of the number of plants that emerged.

All 12 cultures tested were highly pathogenic. There was some variation among the various isolates but it seems likely that this was due to experimental error. It is apparent that the virulence of the 3*A* cultures was not diminished as compared with fresh isolates by growth in culture for a year. This is in marked contrast with the behaviour of Culture 1*A*. In the case of 2*A* (Table II) no loss of pathogenicity was detected by this technique but the wild type was no longer present.

From this experiment the following conclusions may be drawn: A number of wild type cultures of different origin and different cultural history have been shown to possess a high and fairly uniform degree of pathogenicity. Further, no appreciable loss of pathogenicity or change of cultural form as compared with fresh isolates has been found to result from continuing the organism in culture for a period of one year.

Thus, loss of pathogenicity in wild type isolates may be prevented by maintaining the cultures in a pure state and the monosporous technique provides a convenient means of accomplishing this.

(ii) Comparison of the Pathogenicity of Duplicate Subcultures

Eight months after Culture 1*A* was isolated, duplicate subcultures on potato dextrose agar slants were made by the mass transfer method and these were termed 1*A*(1) and 1*A*(2). A monosporous analysis of the contents of these slants performed 12 days later showed that in 1*A*(2) the dark appressed type predominated. In 1*A*(1) the light appressed and intermediate appressed types predominated but one spore among a total of 28 gave rise to a dark semiraised culture. A pathogenicity test showed 1*A*(1) to be more virulent than 1*A*(2), which, in the light of the cultural analysis, is what would be expected. In this test, flasks of cornmeal–sand medium were inoculated with mass transfers of the two cultures. After 10 days flats containing 10 litres of 'sandy knoll' soil were inoculated with the medium in the proportion of 2% by volume and 140 seeds were planted per flat. Culture 1*A*(1) caused 26% pre-emergence blight and 1*A*(2) caused 11%. Since wilting was found to be so severe throughout this set of experiments that very few seedlings remained alive after 15 days, the disease incidence was estimated by deducting the percentage emergence in the inoculated flats from that in the checks. Emergence was found to be consistently low in the case of virulent cultures. In a pathogenicity trial performed simultaneously with this experiment, the pure wild type caused 54% pre-emergence blight whereas a light appressed mutant caused only 13%.

Isolations were made from diseased seedlings in the flats inoculated with 1*A*(1) and 1*A*(2). The pathogenicity of these was then compared in an experiment the technique of which differed from the last in that smaller flats containing three litres of soil were employed. Seventy-five seeds were planted per flat. The results of this experiment are summarized in Table IV.

Cultures 2*K*, 2*L*, 2*M* were isolated from diseased plants inoculated with 1*A*(1) in the previous experiment and 2*P*, 2*Q*, 2*S* were isolated from plants in the same experiment from the flat inoculated with 1*A*(2). In addition the table includes a pathogenicity test of four purified strains of the organism for comparison. A cultural test was performed with the six isolates but since they were not purified by the monosporous technique previous to this, it was difficult to be certain about the cultural types. Cultures 2*L* and 2*P* appeared to be light appressed and 2*S* dark appressed. Cultures 2*K*, 2*M*, and 2*Q* appeared to vary between the light appressed and intermediate appressed.

TABLE IV

COMPARISON OF VIRULENCE OF DUPLICATE SUBCULTURES OF *Fusarium* WITH REISOLATES FROM DISEASED SEEDLINGS. FOUR PURIFIED STRAINS ARE INCLUDED FOR COMPARISON

Culture	Emerged, %	Pre-emergence blight, %	Culture	Emerged, %	Pre-emergence blight, %
1*A*(1)	76	8	Wild type	23	61
2*K*	61	23	Dark semiraised	27	57
2*L*	63	21	Intermediate appressed	32	52
2*M*	69	15	Light appressed	78	6
1*A*(2)	81	3	Check (*a*)	84	—
2*P*	77	7	Check (*b*)	82	—
2*Q*	69	15	Check (*c*)	87	—
2*S*	77	7			

The results indicate, again, that 1*A*(1) was more virulent than 1*A*(2). Further, the isolates from 1*A*(1) were more virulent than those from 1*A*(2). The isolates were more virulent than the parent cultures and this was especially marked with 1*A*(1).

From these experiments it seems reasonable to conclude that, in addition to the hazard of losing the original wild type, the mass transfer method of maintaining cultures favours variation in the type of mutant that predominates. It is evident that such a technique might easily lead to erroneous conclusions regarding the variation in pathogenicity of the organism. For example, from Table IV it might be concluded that passage through the host led to an increase in the virulence of the organism. A more reasonable explanation in view of the preceding work is that the flats from which the cultures were isolated had been inoculated with mixed cultures and that the more virulent strains in the mixture had greater success in attacking the host. Hence they predominated in the diseased plants from which the isolations were made.

(iii) Comparison of the Pathogenicity of Mutants in Sterilized and Unsterilized Soil

In order to approximate more closely the field environment, a set of pathogenicity trials was performed in unsterilized soil. Flats of sterilized and unsterilized 'sandy knoll' soil were inoculated with a 1% concentration by volume of cornmeal–sand medium on which strains of the organism had grown for 10 days. Six type strains were employed: the wild type, two dark semiraised strains, intermediate appressed, light appressed, very light appressed. One hundred and forty seeds were planted per flat.

The wilt progress curves obtained in this experiment have been plotted in Fig. 10. It is noted that in all cases disease incidence was less severe in

unsterilized soil, an effect that is considered to be the result of an unfavourable biological balance that may be removed through sterilization. This problem will be dealt with more fully in a subsequent publication.

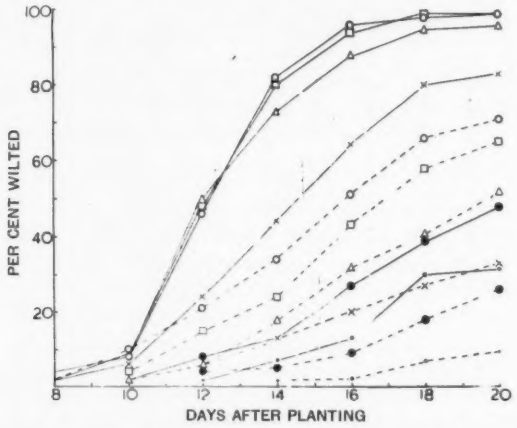


FIG. 10. *Comparison of the pathogenicity of the wild type and five derived mutants in sterilized and unsterilized soil.* O———O *Wild type.* □———□ *Dark semiraised—1.* △———△ *Dark semiraised—2.* ×———× *Intermediate appressed.* •———• *Light appressed.* •———• *Very light appressed.* ——— *Sterilized soil.* — — — *Unsterilized soil.*

The order of virulence of the six strains was found to be similar in both kinds of soil but some differences were evident in the degree of depression of virulence by unsterilized soil. For instance, the disease incidence curves in sterilized soil for the wild type and the first dark semiraised culture (*DSR*-1) were closely parallel but in unsterilized soil, *DSR*-1 was depressed more than the wild type. *DSR*-2 was almost as pathogenic as the wild type in sterilized soil but in unsterilized soil it was depressed to a much greater degree. Similar large depressions were noted with the intermediate appressed and the light appressed but these cannot be compared with the wild type on the same basis as *DSR*-1 since they were considerably less pathogenic in sterilized soil.

Since even the most pathogenic of the mutant cultures was found less virulent in unsterilized soil than the wild type, it is possible that, although these mutants are more aggressive in culture, the wild type is more successful in the field environment.

(iv) Comparison of the Virulence of Mutant Strains under Field Conditions

A field experiment was performed to compare the virulence of some type strains under field conditions. The technique employed for planting the melons was similar to that employed commercially. Four-inch pots of sterilized greenhouse compost soil ($\frac{1}{2}$ loam, $\frac{1}{4}$ sand, $\frac{1}{4}$ leaf mould) were planted

with four seeds per pot. Twenty days later these were thinned to two plants per pot and 10 days after this the melons were transplanted to the field. Inoculum of the organism was prepared by growing the six strains employed in the preceding experiment on cornmeal–sand medium for 10 days. The inoculum was in each case diluted to 2% with sterilized compost soil. Then, as the melons were transplanted from the pots to the field, 200 cc. of the diluted inoculum was sprinkled over the outer surface of the ball of soil. Excess inoculum was allowed to fall into the shallow hole in which the plants were then placed. Thirty-one pots were sprinkled in transplanting with sterilized soil and these served as checks. Eighteen were inoculated with the wild type and 10 with each of the following strains: dark semiraised—1, dark semiraised—2, intermediate appressed, light appressed, very light appressed. The melons were planted in July, 1943. The soil type of the field was Vineland Fine Sandy Loam.

The commonest symptom besides wilting was a marked tendency for the plants to become stunted. Growth was slow with the production of short internodes and small leaves. The stunting was greatest in the case of the more virulent mutant cultures. It was also severe in the plants inoculated with the wild type but most of these wilted so early that comparison was not possible.

Four weeks after transplanting, a survey of the disease incidence was made. Each plant was graded as follows: 0 = normal as exemplified by the checks, 1 = definitely stunted, 2 = severely stunted, 3 = killed. The following values represent the average for each strain of the organism: wild type, 2.9; *DSR*-1, 2.3; *DSR*-2, 2.1; intermediate appressed, 2.2; light appressed, 1.9; very light appressed, 1.2; checks, 0. In four weeks all but one of the plants inoculated with the wild type had wilted. Of those inoculated with *DSR*-1, 40% wilted within this time and in two months 64% were wilted. All cultural types except the very light appressed were reisolated from the diseased plants.

Under field conditions, therefore, the wild type was much more virulent than any of the mutant cultures tested. This suggests that for some reason the wild type is more successful in the field than the mutants. In the preceding experiment it was noted that the virulence of the mutants was more depressed in unsterilized soil than that of the wild type.

During the summer of 1944, the following year, this field was replanted,* the same muskmelon variety being employed and the same technique except that no inoculum was added. Wilt was observed, and from 20 diseased plants a total of 72 isolates was obtained, all of which were of the wild type. None of the mutants was reisolated.

It has been mentioned previously that all fresh isolates from plants diseased in naturally infested soil were found to be of the wild type, a fact that seems remarkable in view of the variation shown by the organism in culture. The

** Through the co-operation of the Horticultural Experiment Station, Vineland, Ont. Isolations were made by the writer from diseased specimens forwarded to the Dominion Laboratory of Plant Pathology, Harrow, Ont., through the courtesy of Professor D. L. Bailey.*

above observations are considered to offer at least a partial explanation for this. It is possible that the mutants, if they do arise in nature, are less effective than the parent in competing with the soil microflora and hence they would tend to be crowded out.

(C) Induced Mutations

(i) Heat

Efforts to induce mutations by heat treatment were unsuccessful. The thermal death point of spore suspensions in test-tubes immersed in a water-bath for 10 min. was found to lie between 45° and 50° C. When the spore suspensions were spread over the surface of agar plates for monosporous analysis, no reduction in germination as compared with the check was found at 45° but at 50° only seven spores germinated. None of the latter differed from the wild type. Also, 56 single spores isolated from the 45° tube all produced wild type cultures.

(ii) Ultra-Violet Light

(a) Standardization of Technique

It has been found possible to induce mutations by means of ultra-violet rays from a Westinghouse Sterilamp *WL*-782-30. In the specifications for this lamp it is stated: "Over 80% of the radiant energy produced by the Sterilamp is in the region of 2537 Angström Units, which provides the most effective bactericidal energy for the power consumed." During the irradiation experiments the lamp was 22 in. above the material treated.

Since Das Gupta (5) has reviewed the subject of induced mutation in fungi, no attempt will be made to do so here. Most of the irradiation experiments in this work were performed with spores that were spread over the agar surface in a Petri dish as in the technique for monosporous isolations. The lids of the Petri dishes were removed during irradiation. The spores were then allowed 10 to 20 hr. to germinate and monosporous isolations were made in the usual manner.

Preliminary experiments were performed to determine (*a*) the optimum period of irradiation for the induction of mutation and (*b*) the time that should elapse between the spreading of the spore suspension over the agar and irradiation in order to secure the maximum amount of mutation.

In the first of these the value of (*b*) was set at two hours and the plates were irradiated for periods of 0 sec., 10 sec., 30 sec., 90 sec., 4 min., 10 min. An average of 15 single spores was isolated from all except the 10-min. plate. In the latter the lethal effect of the rays was so great that no germinating spores could be found, although a number of cultures appeared in this plate a few days later. Three of the 16 spores isolated from the four-minute plate produced mutant cultures but none were obtained from any of the other plates. In the next experiment the value of (*a*) was set at five minutes and (*b*) was varied. Periods of zero, one, two, four, eight hours were employed but no correlation was evident between the value of (*b*) and the amount of mutation. Two hours was selected arbitrarily as the standard time.

The next step was to determine more accurately the optimum period of irradiation. Plates were irradiated for zero, one, two, three, four, five minutes. Forty germinating spores were isolated from each plate but it is evident from Table V that not as many of these survived at the longer periods of irradiation. The reason for this low survival is not clear. Longer treatment may have rendered the spores more sensitive to the disturbance of transferring. Germination was, on the average, much more rapid in the check plate. It was noticeably slower at two and three minutes and much slower at four and five. Since the highest proportion of mutant cultures was obtained at four minutes, this was adopted as the standard period of irradiation.

TABLE V

THE EFFECT OF PERIOD OF IRRADIATION ON THE INDUCTION OF MUTATIONS IN *Fusarium* SPORES

Period of irradiation, min.	Number of single spore cultures obtained from 40 transfers	Number of mutant cultures
0	39	0
2	37	3
3	35	2
4	29	4
5	27	3

(b) Description of the Ultra-violet Mutants

No specific features have been observed in the ultra-violet mutants that have not also been noted among the 'natural' mutants. A few general differences have been noted. For instance, a greater proportion of the ultra-violet mutants appear to be of a slow-growing, weak type. This seems not unexpected since the natural mutants arise in culture and such forms would tend to be crowded out. Most of the induced mutants that are dark seem, on the average, much darker than similar natural mutants. They also tend to have more aerial mycelium than the natural dark types. Many of the mutants were similar to some of the natural mutants. For example, several were indistinguishable from the light appressed type already described. Some ultra-violet mutants are shown in Fig. 8.

(c) Induced Mutations in Cultures Other Than the Wild Type

It has been found possible to induce mutations in the dark semiraised and the intermediate appressed mutant types. In the former, one mutant was induced among 22 spores isolated and in the latter, two mutants per 29 spores. Under the same conditions, four mutants appeared per 38 spores in the wild type. No mutant cultures were obtained with the light appressed and very light appressed although a total of 62 cultures was grown from irradiated spores.

Spores from five ultra-violet mutants were irradiated to determine whether further mutation could be induced. From two of these, which were classified as dark raised, a total of three mutants was obtained, one of which was light appressed.

The variability of this organism seems consistent in one respect. The mutants appear to be unidirectional. That is, they tend to change from the light raised or wild type to a dark raised or dark semiraised and then to light appressed. This has been observed to happen in three ways: (i) both mutant steps have occurred naturally; (ii) the first has been induced and the second has occurred naturally and vice versa; (iii) both steps have been induced by successive irradiations. The intermediary dark stage may be passed over and the change from wild type to light appressed occur in one step. This has frequently occurred naturally and it has also been induced. No evidence of reversion was ever obtained.

(d) The Virulence of Ultra-violet Mutants

A pathogenicity test with nine ultra-violet mutants was performed in conjunction with the experiment outlined in Table III and the results are hence comparable. Six wild type cultures were included (the 4*M* cultures). The spores that produced these cultures were irradiated for four minutes but no visible change resulted.

Before the flasks of cornmeal–sand medium were inoculated, all 15 of these cultures were purified by the monosporous technique.

The virulence of Cultures 1 to 5, as shown in Table VI, appears very low. This is particularly striking when compared with that of the wild types in Table III. Nos. 2 and 4 were weakly-growing cultures and low virulence

TABLE VI

COMPARISON OF THE VIRULENCE OF MUTANTS INDUCED IN THE MUSKMELON WILT *Fusarium* BY ULTRA-VIOLET IRRADIATION

Number of culture	Wilt in 15 days, %	Wilt in 18 days, %	Number of culture	Wilt in 15 days, %	Wilt in 18 days, %
1	0	0	9	78	95
2	0	1	10	72	90
3	2	6	4*M*—1	69	86
4	3	4.5	4*M*—2	90	98
5	16	25	4*M*—3	2	5
6	29	59	4*M*—4	92	99
7	39	64	4*M*—5	90	98
8	73	89	4*M*—6	83	98

might have been expected but Nos. 1, 3, and 5 grew well in culture. No. 1 was light appressed and Nos. 3 and 5 were both dark raised. Cultures 6 to 9 were more virulent than 1 to 5 but did not equal the wild types. It is remarkable that Culture 9 (light appressed), which arose spontaneously as a mutant from Culture 8 (dark raised), appeared to be more virulent than its parent

but the virulence was still lower than that of the wild type. In no other instance was a mutation found associated with a gain in pathogenicity. Culture 10 was a natural mutant (light appressed) included here for comparison. It was more virulent, apparently, than either of the induced light appressed cultures (Nos. 6 and 7). All the ultra-violet mutants except No. 1 were reisolated from diseased seedlings and had, apparently, not been altered by passage through the host.

It is interesting to note that two of the 4*M* cultures, 4*M*-1 and 4*M*-3, appeared less virulent than the other four, which compared favourably with the wild types in Table III. Culture 4*M*-3 was particularly low in pathogenicity. Thus it seems possible to induce changes in pathogenicity without affecting the cultural form. This indicates that the factor or factors governing pathogenicity are not identical with those for cultural form.

(e) Apparent Reversion

Mutant cultures arising from irradiated single spores have occasionally been observed to revert to the parent. The centre of such a culture may be markedly different from the margin, which begins to resemble that of the wild type. This observation suggests a reversion but further investigation has shown that these cultures were initially impure. When mutant cultures were purified by the monosporous technique, no evidence of reversion followed.

The conclusion seems reasonable that, when a mutation is induced in one cell of a two-celled spore, for instance, a mixed culture would result if both cells germinated. Then, if the mutant component is not able to compete with the wild type, the latter soon predominates and the phenomenon of apparent reversion is observed. The fact that this effect was infrequent is readily correlated with the infrequency of multicellular spores in the wild type.

(f) The Effect of Irradiating Growing Cultures

The effect of irradiating growing cultures of the wild type is striking (Fig. 9). In about two days a deep greyish-purple colour is noted in the central area of the culture and this appears similar to the effect reported by Gier (6) who found that irradiation of cultures of *Gibberella Saubinetii* (Mont.) Sacc. with a mercury vapour lamp caused the cultures to darken from pale cream to purple-black. In a week or more a ring made up of dense patches of raised mycelium marks the circumference of the culture at the time of irradiation. Appressed mutants have been isolated from these patches by the single-spore method. Microscopic examination showed immense quantities of chlamydospores to occur in this ring. These are seldom produced by the wild type on potato dextrose agar but certain mutants form them profusely.

It seems, therefore, that these dense patches are analogous to the dense patches that arise spontaneously in wild type cultures and that have been found associated with the appearance of mutations. It is likely that the advancing edge of the culture is especially sensitive to irradiation since it is not shaded by aerial mycelium, or the age of the mycelium may be involved,

with the result that a large number of mutations are induced there. Those that are sufficiently aggressive to invade the surrounding wild type proceed to do so and as a result the ring of dense patches is produced. A smaller number of these appear within the ring.

When cultures other than the wild type were irradiated, the results were not as striking. Only in the dark semiraised was a darkening effect noted. In all, however, a definite ring marked the circumference of the cultures at the time of irradiation. This was very faint in the very light appressed but in the dark semiraised, intermediate appressed, and light appressed it was quite sharp, although no dense patches appeared on it.

3. Experiments on Cultural Degradation

(A) The Effect of Frequency of Transfer on the Purity of Cultures

The process termed cultural degradation has been described under 1 (above) and its importance in regard to cultural and pathogenicity studies with the organism has been emphasized under 2. The effect of varying the cultural technique upon the rate of this process has been studied in the hope of finding a method whereby the hazard could be diminished or avoided completely.

The first of these experiments involved varying the frequency of transfer. Two wild type cultures, 3*A*-4 and 1*D*, were selected. Culture 3*A*-4 was isolated in 1942 and was maintained in the pure state by four successive monosporous isolations. Culture 1*D* was isolated in 1943, two weeks before the experiment was begun.

Nine slants of potato dextrose agar were inoculated with each of these cultures. During the succeeding six months a group of three slants from both was transferred 13 times. A second group was transferred twice and a third was not transferred at all. All slants in the last group were sealed with Parafilm one month after inoculating to prevent drying. Each time the second group was transferred, an average of 20 monosporous isolations was made from each of the cultures in the slants that were transferred. At the same time similar isolations were made from the first set. Finally, after six months an average of 40 single spores was isolated from each of the 18 slants. The results are summarized in Table VII. Each of the figures represents the average of three slants.

Two main conclusions may be drawn from this table. (i) Successive purifications of Culture 3*A* by the monosporous technique did not render it more stable than the recent isolate 1*D*. No consistent differences were observed between the two. (ii) Frequent transferring definitely accelerates the rate of cultural degradation. This was first evident after four months when Group 1 had been transferred eight times and Group 2 once. Failure to transfer at all as in Group 3 definitely favoured the wild type.

There appears to be a limit to the accelerating effect of frequent transfers on cultural degradation. From the advancing margin of a wild type culture, 16 successive transfers were made in Petri dishes at intervals of three to five

TABLE VII

COMPARISON OF THE EFFECT OF FREQUENCY OF TRANSFER ON THE PURITY OF TWO CULTURES OF THE WILD TYPE OF THE MUSKMELON WILT *Fusarium*

Group	Total transfers in 6 months	Culture	Percentage of mutants isolated after:		
			2 months	4 months	6 months
1	13	3*A*—4	32	100	100
		1*D*	71	100	100
2	2	3*A*—4	70	79	100
		1*D*	37	63	69
3	0	3*A*—4			25
		1*D*			33

days, and no change of cultural form was observed. Hence it is likely that some feature associated with older cultures is involved. The dense mutant patches that appear in cultures have already been described. These gradually increase in size, requiring usually two weeks to become prominent. They occur in slants and it seems probable that if serial transfers were made with this interval, the mutants would soon predominate.

A large variety of mutant types was obtained in this experiment, the commonest of which was the light appressed. This type predominated in eight slants out of 12. The dark semiraised predominated in two and the dark appressed and very light appressed in one each. The mutants that appeared in Group 3 (not transferred) were unique in the fact that many resembled the parent wild type quite closely. Since none of these were found in the transferred slants, it is likely that they are no more able to withstand the competition of the appressed types than the parent and disappear simultaneously.

An obvious conclusion of practical importance may be drawn from this experiment. To maintain the wild type culture in as pure a state as possible, transfers should be infrequent and, preferably, associated with monosporous purifications.

(B) THE EFFECT OF VARYING THE CULTURAL MEDIUM ON CULTURAL DEGRADATION

All previous work was performed on potato dextrose agar. The possibility that the organism might be more stable on other media was investigated. Eight types of nutrient medium were prepared as follows.

Potato dextrose agar: prepared according to the formula of Riker and Riker (14), except that the concentration of agar was 1.5% instead of 1.7%. All the following media were made up with 1.5% agar. Eight slants of potato dextrose agar were included in this series, four of which were sealed with Parafilm after inoculating and this was repeated after every transfer.

Potato agar without dextrose: same as the preceding but with no added dextrose.

Malt, pea, and beef-extract–peptone agars: prepared according to formulae of Riker and Riker.

Synthetic nutrient agar: prepared by mixing equal volumes of agar solution and a nutrient solution the composition of which was that of Pfeffer's except that dextrose was added to make a final concentration of 2% in the agar.

Soil agar: an equal volume of agar solution was mixed with the supernatant liquid obtained by suspending 'sandy knoll,' soil in an equal volume of water and allowing the heavier grains to settle.

Potato slices: under sterile conditions slices 2 to 3 cm. long and ½ to 1½ cm. thick were cut from a large potato and placed in sterilized test-tubes containing 1 to 2 cc. of sterile water.

Visible growth of the organism occurred in all tubes except those containing soil agar. On this agar the wild type grew very sparsely with no aerial mycelium. However, the rate of radial growth in Petri dishes observed with a dissecting microscope was approximately the same as on potato dextrose agar. The addition of 2% dextrose to soil agar increased the growth rate slightly but did not cause aerial mycelium to appear. Growth was also rather sparse on the potato slices and beef-extract–peptone agar. Sporulation was most abundant on potato agar without dextrose and numerous three-septate macrospores were produced. Chlamydospores were formed in soil agar but were very infrequent in the other media.

Four tubes of each nutrient type were employed and these were inoculated with a loop of spore suspension from a wild type culture that had previously been purified 10 successive times by the monosporous technique. In the succeeding 12 weeks, nine transfers were made at intervals of one to two weeks, following which an average of 40 single spore isolations was made from each tube. The results of these isolations are summarized in Table VIII.

The cultures that predominated in most tubes were the light appressed and dark semiraised types. No marked variation between the types of mutant

TABLE VIII

MONOSPOROUS ISOLATIONS FROM TUBES OF NUTRIENT MEDIUM INOCULATED WITH THE WILD TYPE AND TRANSFERRED NINE TIMES

Type of medium	Mutants isolated after 12 wk., %			
	Tube 1	Tube 2	Tube 3	Tube 4
Potato dextrose agar	100	100	100	100
Potato dextrose agar, sealed	0	0	100	100
Potato agar	0	0	100	2
Malt agar	100	63	100	100
Pea agar	66	100	100	100
Beef-extract–peptone agar	100	100	100	100
Synthetic nutrient agar	100	0	100	0
Soil agar	0	0	0	0
Potato slices	4	0	100	100

that appeared on the different media was noted except in the case of the potato slices. The mutant that crowded out the wild type in two of these tubes was closely similar to the parent but differed noticeably in the character of the aerial mycelium. No similar mutants were observed throughout the series.

Cultural degradation appeared complete on potato dextrose and beef-extract–peptone agars and almost complete on malt and pea. It is remarkable that on soil agar no evidence of cultural degradation was obtained. Sealing potato dextrose slants with Parafilm had the effect of hindering cultural degradation in two of the tubes. Growth was somewhat more profuse in the unsealed tubes but it is difficult on this basis to account for the effect of sealing on cultural degradation. The fact that the isolates from a given tube were either 100% or 0% mutants suggests that a hindrance to mutation is involved. If the effect were a retardation of the crowding-out phase, then mixtures of the wild type and mutants would be expected. By failing to add dextrose to potato agar, cultural degradation appears to have been retarded, which may be associated with the absence of a readily available nutrient. This is supported by observations on soil agar and potato slices where readily available nutrients would likewise be limited. In the case of the sealed potato dextrose agar slants, it is possible that the organism was unable to use the dextrose readily because of oxygen lack. However, the synthetic agar contained 2% dextrose yet here, too, the loss of the wild type was retarded.

The nutrient factor responsible for instability is hence difficult to assess from this experiment but the possibility that the presence of readily available nutrient favours breakdown is indicated. The experiment suggests that soil or soil agar may be of value as nutrient media for maintaining Fusaria in culture.

(C) Stability in Soil and Soil Agar

In (B) a remarkable degree of stability of the organism in soil agar was evident. Similar observations have been made in connection with an experiment on the rate of growth of the wild type through tubes of soil. Eighteen months after this experiment ended, isolations were made from the eight test-tubes and four types of soil involved by dusting soil from each tube finely over the surface of an agar plate. Hyphal tips that emerged from the granules were isolated by means of the 'biscuit cutter' technique. Two out of 276 cultures thus obtained differed from the wild type in the colour of the submerged mycelium, which was very dark. The aerial mycelium resembled that of the wild type, however.

A subsidiary experiment in this regard was performed in connection with the experiment that studied the effect of frequency of transfer on cultural degradation. Six tubes of sterilized 'sandy knoll' soil were employed. These were inoculated with each of the two wild type cultures involved and were transferred twice in six months. The transfers were accomplished by inoculating fresh tubes with soil from the lower parts of the previously inoculated

tubes. After six months five of the tubes yielded only one mutant (dark raised) per 190 cultures obtained by the 'biscuit cutter' technique. From the other were obtained 25 wild type cultures and 15 that were dark semiraised. These resembled mutant types that appeared on potato dextrose and other agars. This tube constituted such an exception that it seems likely that the mixture was introduced with the inoculum. At the time the significance of the 'patch mutants' was not realized and inoculum for this tube may have been taken from such an area.

Two explanations for the stability of the wild type in soil agar seem possible. (i) Mutations do not occur in soil agar, or (ii) mutations do occur but are less aggressive on this medium and are crowded out by the wild type. This was investigated further by inoculating slants of soil agar with a mixture of spore suspensions of the wild type plus a light appressed mutant. The composition of the dilutions was determined by isolating an average of 40 single spores from each. After seven transfers on soil agar at intervals of six to 10 days, the cultural content of each slant was analysed by the same method. The series was performed in triplicate.

The data obtained are summarized in Table IX. It is evident that the wild type can be crowded out by even a very low initial concentration of the light appressed mutant. It is thus apparent that this mutant is more aggressive on soil agar than the parent wild type.

TABLE IX

MONOSPOROUS ISOLATIONS FROM SLANTS OF SOIL AGAR INOCULATED WITH MIXTURES OF WILD TYPE PLUS MUTANT AFTER SEVEN TRANSFERS. AN AVERAGE OF 30 SPORES ISOLATED PER SLANT

Initial concentration of mutant, %	Concentration of mutant after seven transfers		
	Tube 1	Tube 2	Tube 3
0	0	0	0
2	0	52	100
10	97	93	100
64	100	100	100
100	100	100	100

This points to the conclusion that the failure of the light appressed or of other mutant types to displace the pure wild type in soil agar is due to the infrequence or absence of such mutations in this medium.

4. CULTURAL INTERACTION

(A) DESCRIPTION

When a culture of the wild type is grown in the same Petri dish with, for example, a light appressed mutant, the latter appears to invade the wild type along the line of contact. An instance of this is shown in Fig. 11. Here four Petri dishes were inoculated in the centre with a light appressed culture

and midway between this point and the edge inoculum of five cultures of the wild type was placed. These were placed near a window and two were wrapped in a dark cloth. The photograph, which includes one of each, was taken 18 days later. A dense white zone of mycelium appeared within the margin of the wild type cultures where they contacted the mutant and this effect was more striking in the darkened plates. The colour of the submerged mycelium beneath these dense white areas resembled the colour of the mycelium beneath the 'patch mutants' already described and tended to be a dark greyish-purple. Monosporous isolations from this dense zone showed that the light appressed mutant predominated here. Only four spores out of 70 produced wild type cultures.

A similar effect was noted by Brown (1) in the *Fusarium* with which he worked, where an intensely coloured ridge appeared between sectors and adjacent normal areas of some cultures. Wellman (16) grew cultures of a virulent isolate of *Fusarium bulbigenum* var. *Lycopersici* (Brushi) Wr. & R. in various associations with a dark appressed culture and in certain cases, as shown in his Fig. 1, p. 183, this effect is clearly evident, although no reference to it is made in the text. These observations indicate that it is not an effect peculiar to the muskmelon *Fusarium* only.

This phenomenon has been termed 'cultural interaction.' It appears to involve an actual invasion of the wild type culture by the mutant. This is indicated by the fact that the light appressed can be isolated from within the original margin of the wild type cultures. The dark semiraised type is quite as effective as the light appressed in producing this effect and in this instance the dark semiraised can be isolated from the dense zone. This adds further support to the view that interaction is the result of an invasion by the mutant. The alternative view, that the mutants secrete a substance that diffuses into the wild type culture and there induces mutation, requires that the substance be so specific that it induces the kind of mutant that produced it. The invasion hypothesis seems more reasonable. The light zone that appears within the margin of the light appressed (Fig. 11) may not be due to a reverse invasion by the wild type since 65 single spore cultures were obtained from this zone and all were of the light appressed type. It may be caused by substances that diffuse in from the wild type or are produced by the light appressed where it contacts the wild type.

It is likely that the increase in size of the patch mutants and cultural interaction are analogous and represent different expressions of the same phenomenon, the ability of certain mutants to invade cultures of the wild type.

(B) Experiments on Cultural Interaction

(i) The Interaction of other Mutant Types with the Wild and the Light Appressed Types

Eleven mutants were employed and were divided into two groups: (i) six mutants much different from the wild type. Three of these were dark semi-

raised, one was light appressed, one intermediate appressed, and one dark appressed; and (ii) five mutants that closely resembled the wild type.

All the cultures in Group (1) produced cultural interaction where they contacted the wild type but this was not evident where they contacted the light appressed. One of the cultures in Group (2) showed interaction with both the wild type and the light appressed. Two of the others interacted slightly with the wild type only. The remaining two showed no evidence of interaction with either the wild type or the light appressed.

(ii) The Interaction of Wild Type Cultures from Irradiated Spores with the Light Appressed and Wild Type

On Page 32 the virulence of six wild type cultures that had arisen from irradiated spores was studied. It was found that two had become less virulent. Five of these cultures were further studied to determine whether this treatment would affect cultural interaction. All five cultures were first purified by the monosporous method and were then grown in Petri dishes with the wild type and the light appressed. All showed cultural interaction with the light appressed but not with the wild type, indicating that the irradiation had not disturbed the ability of the wild type to interact with the light appressed.

(iii) Interaction Between the Light Appressed and Other Fusaria

To determine whether interaction was a specific phenomenon between parent and mutant, the light appressed strain was allowed to contact a number of other Fusaria in culture. A representative plate from the first of these tests is shown in Fig. 12. In this instance *A* is a culture of the wild type and *B* a light raised culture obtained by monosporous analysis from a recently isolated wild type culture. Though it may have existed as such in the field, it is possible that it arose as a mutation in culture. Nos. 1 and 2 were Fusaria isolated from soil. Between the light appressed and *A*, the parent culture, a row of dark spots was apparent which, when viewed from above were a dense white. Between the light appressed and Culture *B* this did not appear but instead a sharp line of demarcation resulted. No. 2 appeared to invade the light appressed and it was found possible to isolate it from within the margin of the latter. Between No. 1 and the light appressed

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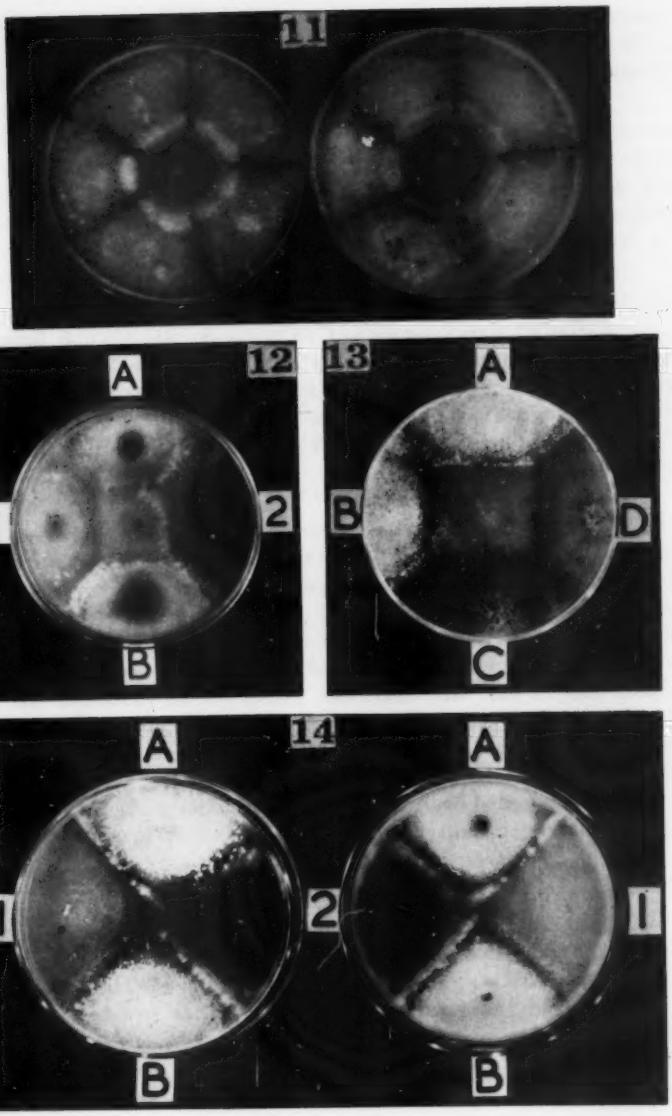
EXPLANATION OF PLATE II

FIG. 11. *Cultural interaction between a light appressed mutant and five isolates of the wild type of the muskmelon wilt* Fusarium. *The photograph was taken 18 days after inoculating, during which period the plate to the left was kept in the dark.* FIG. 12. *Cultural interaction between four Fusaria and a light appressed mutant obtained from the muskmelon wilt* Fusarium. *A = Muskmelon wilt* Fusarium. B = *Mutant (?) isolated from A. 1 and 2 = unidentified Fusaria isolated from soil by the plate method. The plate is inverted.* FIG. 13. *Four pathogenic Fusaria matched with a light appressed mutant of the muskmelon wilt* Fusarium. *A = Muskmelon wilt* Fusarium. *B = Tomato wilt* Fusarium. *C* = Gibberella sp. *D* = Fusarium moniliforme *Sheldon.* FIG. 14. *Cultural interaction between two Fusarium wild types and derived mutants. A = Muskmelon wilt* Fusarium. *B = Tomato wilt* Fusarium. *1 = Light appressed mutant from A. 2 = Dark appressed mutant from B. The plate to the right is inverted.*


11
A
12
1
2
B
13
A
B
D
C
14
A
A
1
2
1
B
B

no line of separation or evidence of invasion was noted. The two cultures appeared compatible. It is thus evident that, within the limits of this experiment, cultural interaction with the light appressed was confined to the parent.

Fig. 13 shows the results of a similar experiment in which the light appressed was allowed to contact the parent wild type (*A*) and three pathogenic Fusaria recently isolated from diseased material. These were (*B*) *Fusarium Lycopersici* Brushi, (*C*) *Gibberella* sp. from corn, and (*D*) *Fusarium moniliforme* Sheldon, also from corn. Cultural interaction, again, was evident only between the light appressed and its parent wild type.

In Fig. 14 the specific nature of cultural interaction is further indicated. An appressed mutant was obtained by the monosporous technique from a 'patch mutant' that appeared in a monosporous culture of *Fusarium Lycopersici* Brushi. The two wild types and their derived appressed mutants were matched as shown. Between each mutant and its parent interaction was noted but this did not appear between it and the other wild type.

(C) The Taxonomic Value of Cultural Interaction

In the preceding experiments specificity in regard to cultural interaction was evident in two ways: (i) among mutants derived from the same parent some interact with the parent and some do not; (ii) cultural interaction between the wild type and its derived light appressed differed from the type of interaction noted between the latter and a number of other Fusarium isolates. It is possible that these observations, especially (ii) may be of some value in the taxonomy of the genus *Fusarium*. In view of the difficulty experienced in classifying the Fusaria on a morphological basis, an effort has been made by Coons and Strong (4) to establish a method of diagnosing *Fusarium* species by means of their physiological reactions to dyes. The possibility that specific reactions of the type described here would be of value in the identification of *Fusarium* species is a question deserving of further attention.

Summary and Discussion

The observations reported in this article indicate that the muskmelon wilt *Fusarium* occurs in nature as a form (or forms) that, when cultured on most artificial media, produces abundant aerial mycelium on which conidia, mostly non-septate, are borne rather sparsely. This form has been termed the 'wild type.' Since the organism was shown to be predominantly uninucleate throughout its life cycle, the conclusion is drawn that cultural variation here is due to mutation rather than the segregating out of genetically different nuclei.

Many of the variants are more aggressive in culture than the parent wild type, in consequence of which they tend to crowd out the latter. This process, which has been termed cultural degradation, is favoured by frequent transfer. Since the variants are, in general, less pathogenic than the parent,

cultural degradation is found associated with a loss in virulence. It may be prevented through frequent purification of the original isolates by the single-spore technique.

Evidence is presented that the wild type is genetically very stable under nutrient conditions approximating those of the soil environment, indicating that, if such variants do appear in nature, they are far less frequent than in the usual laboratory media. Further, the field experiment has suggested that under field conditions mutants of the types that commonly arose in culture were less successful as parasites than the wild type indicating that such variation probably is of little significance in the field pathology of the disease.

If this condition is found to apply generally in the genus *Fusarium*, it is evident that a revision of the basis for taxonomy in the genus would be desirable since it seems only reasonable to base the taxonomic scheme on the types found in nature. The generally accepted view, as outlined by the members of the Fusarium Conference (17) is that macroconidia are to be regarded as the spore type on which species descriptions should be based and that Fusaria that do not produce these when isolated from nature may be induced to do so by frequent transfers. This gives rise to a cultural state characterized by the abundant proliferation of macroconidia, a condition that has been variously called 'normal culture,' 'high culture,' 'Normkultur,' 'Hochkultur.' However, the present studies have shown that the muskmelon *Fusarium* when isolated from nature does not produce macrospores on most media but, when serial transfers are made, it is soon displaced by a mutant such as the light appressed, which bears abundant macrospores. That is, according to Wollenweber *et al.* (17) it would then be in the 'normal' condition from the standpoint of determination. But it seems reasonable that in preparing a species description of such an organism great care should be taken to ensure that the features employed are exclusively those of the wild type. In this case the treatment commonly employed to obtain the so-called 'normal culture' results in the displacement of the wild type by a mutant, which raises the question: is the 'normal' state merely a condition in which the wild type is dominated by a mutant that produces abundant macrospores? If this mutant were always the same the objection might be lessened but it has been shown in the present studies that a great variety of macrospore-bearing cultural types may come to dominate the wild type.

It is important to realize that this situation has been shown to exist in but one member of a very abundant genus. If it is found to be of general application throughout the Fusaria, the conclusion would seem inescapable that most Fusarium descriptions, owing to the technique employed in their preparation, are based on cultural variants of originally pure wild types and a re-evaluation of the taxonomic situation within the genus would be advisable.

At the present time the writer is conducting cultural studies on a number of pathogenic Fusaria with a view to determining whether the 'wild type' concept can be applied more widely in the genus. When this is completed,

a fuller discussion of cultural variability and its relationship to Fusarium taxonomy will be given.

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